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SEPARATION OF THREE GLUCOCORTICOID-BINDING FRACTIONS FROM CYTOSOL OF RAT HEART

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SUMMARY

By chromatography on AcA-34 and AcA-44 ultra-gels three glucocorticoid-binding fractions were separated from heart cytosol preparations of adrenalectomized rats. In several respects the glucocorticoid binding factor of the first fraction resembles glucocorticoid cytoreceptor of liver. At low ionic strength this binding protein is eluted in 7.1 S form whereas at high ionic strength it is dissociated into 5.2 S form. The second fraction contains transcortin, which can be washed out of the tissue by a prolonged perfusion. It apparently contaminates cytosol during tissue homogenization. The third fraction (like transcortin) binds corticosterone but does not bind dexamethasone. It differs from transcortin in chromatographic behaviour and in sensitivity to SH-blocking agents. The three glucocorticoid-binding fractions described are also found in liver cytosol.

INTRODUCTION

It has been reported recently that cytosol from the rat heart contains two glucocorticoid-binding factors—binding factor I and binding factor II [1, 2]. These binding factors can be separately detected in the unfractionated cytosol due to differences in their thermal lability and binding affinity to synthetic glucocorticoid-dexamethasone. Binding factor I was shown to possess several properties of the serum corticosteroid-binding globulin—transcortin. Binding factor II in some respects resembles cytoreceptors of target organs for glucocorticoids.

The aim of this work was to separate binding factor II ("candidate" for the role of glucocorticoid receptor of heart cytoplasm) from other glucocorticoid-binding sites and to estimate the sizes of binding macromolecules by means of chromatography on acrylamide-agarose ultra-gels. The results obtained give additional supports for the resemblance of binding factor II to glucocorticoid cytoreceptors. Binding factor I of the unfractionated cytosol is shown to contain a mixture of at least two glucocorticoid-binding proteins, one of which evidently presents extracellular transcortin.

MATERIALS AND METHODS

Animals. In all experiments "Wistar" male rats weighing 240–250 g were maintained on usual standard laboratory diet. Bilateral adrenalectomy was carried out under light nembutal anesthesia, and the animals were decapitated on the 5th day after the operation.

Steroids. [^{3}H]-corticosterone (specific radioactivity 101 Ci/mmol and [^{3}H]-dexamethasone (specific radioactivity 26 Ci/mmol) were from the Radiochemical Centre Amersham, England. The radiochemical purity of these preparations was checked by t.l.c. on silica gel. Non-labelled steroids were bought from "Sigma", USA.

Heart and liver cytosol preparations. Two different procedures of heart washing to exclude vascular blood transcortin have been used. In one series of the experiments hearts excised from the decapitated animals were kept in 0.9% NaCl for 30 s and then perfused by inserting a syringe through the aorta and washing with the same solution (about 20 ml) at room temperature. In other experiments hearts were isolated by the Langendorf procedure and perfused at 37°C by Krebs–Henseleit buffer solution before isolation of the cytosol. Liver was perfused by syringe through vena cava with the same solution.

All stages of cytosol preparation were carried out at 4°C. Ventricular tissue was separated from atrium and large vessels, cut by scissors into small pieces and washed three times with buffer homogenizing solution. Samples of tissue were treated at minimal speed in a blender type homogenizer. The disintegrated tissue was transferred to a Teflon–glass homogenizer and further homogenized (3–5 strokes) at the ratio of 1:3 (w/v) tissue to homogenizing buffer. The composition of homogenizing buffer was 0.25 M sucrose, 0.05 M KCl, 0.0015 M Na_2 EDTA, 0.0015 M dithiothreithol, 0.01 M Tris–HCl, pH 7.4. The homogenate was centrifuged in a 65 Rotor of Beckman L5-50 centrifuge at 105,000 *g* for 60 min. The precipitate was discarded and the supernatant used in binding experiments.

[^{3}H]-glucocorticoid-cytosol-complexes. To 2 ml of heart cytosol (protein concentration 10–12 mg/ml) or liver cytosol (protein concentration 15–25 mg/ml) 150–200 ng of [^{3}H]-corticosterone or 300–400 ng of

[^{3}H]-dexamethasone was added. The mixture was incubated at 4°C for 4 h. Incubation was stopped by adding 0.2 ml of a 5% suspension of activated charcoal (Norit A) in 0.5% of dextran T-80. After 5 min vigorous stirring, cytosol-bound steroid was separated from the unbound form (adsorbed on charcoal) by centrifugation at 5,000 *g* for 20 min.

Gel-chromatography of [^{3}H]-glucocorticoid-cytosol complexes. Samples containing cytosol-bound [^{3}H]-steroid were applied to acrylamide-agarose ultra-gel columns (0.9 cm mt. dia. 50 cm). Two ultra-gels have been used in these experiments (1) AcA-34 (for the separation of fractions with molecular weights ranging from 20,000 to 350,000 daltons); (2) AcA-44 (ranging from 20,000 to 120,000 daltons). Equilibration of the columns and elution were carried out at 4°C using a buffer solution similar to the homogenizing solution, but containing no sucrose. Collection of eluate was performed automatically on an "LKB" fraction collector fitted with an ultraviolet absorptiometer. Aliquots of fractions (0.1–0.3 ml) were transferred to scintillation vials containing 10 ml of Bray's scintillation cocktail [3]. Radioactivity was measured in a liquid scintillation spectrometer (Nuclear Chicago, Mark 2). Tritium counting was made with 29% effectiveness. All experiments were repeated no less than four times.

RESULTS

In the present work [^{3}H]-glucocorticoid-binding displaceable by excess of non-labelled glucocorticoids is termed "specific" for distinguishing it from non-displaceable low affinity binding.

Gel-chromatography of [^{3}H]-dexamethasone-cytosol complexes

As shown in Fig. 1, [^{3}H]-dexamethasone–heart cytosol complex elutes from the AcA-34 gel column at low ionic strength as a single peak with $R_F = 1.25$ and the profile of radioactivity does not coincide with major protein peaks. Addition to the incubation mixture of, (1) 1000-fold excess of non-labelled dexamethasone, (2) SH-blocking agent, *p*-chloromercuribenzoate (PChMB) in 5 mM concentration or (3) preheating of mixture at 37°C for 20 min led to a sharp "depression" of this radioactivity peak. The "nondepressed" part of radioactivity apparently belongs to a nonspecific complex of [^{3}H]-dexamethasone with higher molecular weight aggregates.

Figure 2 demonstrates that at high ionic strength (0.2 M KCl) specifically bound [^{3}H]-dexamethasone elutes as a peak with lower R_F value (1.65). The peak corresponding to the non-specifically bound steroid becomes larger. This can be explained by a partial transformation of specific complex to non-specific aggregates due to high salt concentration.

Gel-chromatography of [^{3}H]-corticosterone-cytosol complexes

In experiments with cytosol prepared from hearts perfused by a syringe through the aorta specifically bound [^{3}H]-corticosterone was eluted from AcA-34 ultra-gel at low ionic strength as two peaks (Fig. 3). First a relatively small radioactive peak was sharply "depressed" if excess of non-labelled corticosterone or dexamethasone (not shown in the figure) was added to the incubation mixture. Its R_F value was analogous

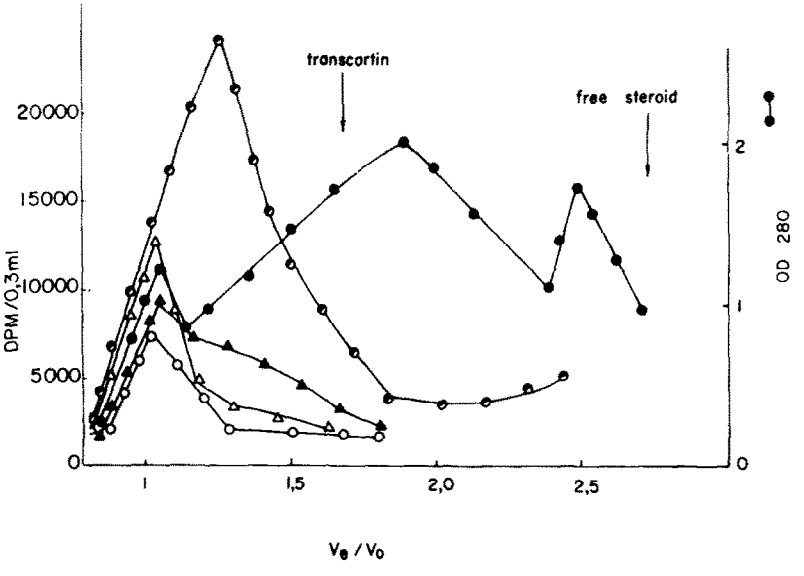

Fig. 1. Elution of [^{3}H]-dexamethasone-heart cytosol complex on AcA-34 ultra-gel column at low ionic strength (0.05 M KCl). ●——● [^{3}H]-dexamethasone ○——○ [^{3}H]-dexamethasone + 1000-fold excess of non-labelled dexamethasone. △——△ [^{3}H]-dexamethasone + *p*-chloromercuribenzoate. ▲——▲ [^{3}H]-dexamethasone + heating at 37°C. Arrows show the position of the serum transcortin peak and the free steroid peak.

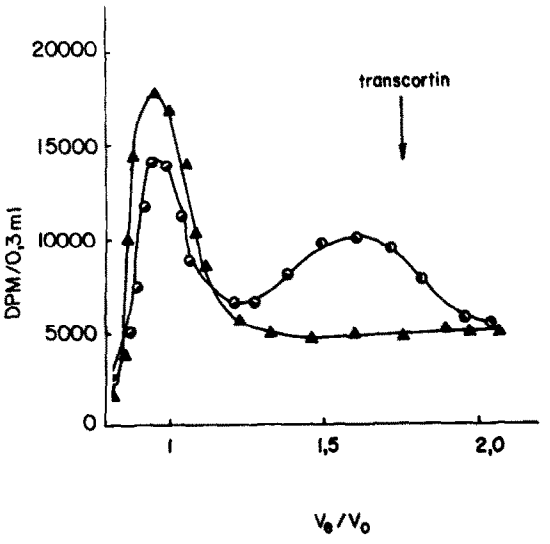

Fig. 2. Elution of [^{3}H]-dexamethasone-heart cytosol complex from AcA-34 ultra-gel at high ionic strength (0.2 M KCl). ◐——◐ [^{3}H]-dexamethasone. ▲——▲ [^{3}H]-dexamethasone + non-labelled dexamethasone.

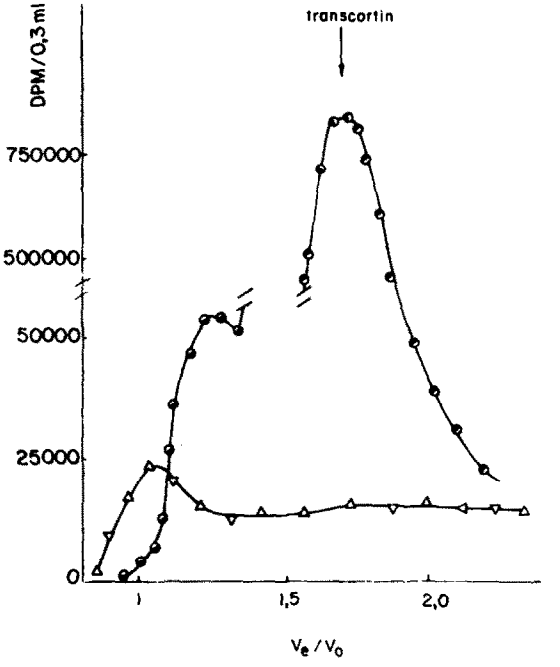

Fig. 3. Elution from AcA-34 ultra-gel of [^{3}H]-corticosterone-heart cytosol complex obtained from hearts washed by syringe through aorta. ◐——◐ [^{3}H]-corticosterone. △——△ [^{3}H]-corticosterone + 1000-fold excess of non-labelled corticosterone.

to that of the [^{3}H]-dexamethasone complex. The second very high radioactive peak was depressed after non-labelled corticosterone treatment. This coincided in R_F with the [^{3}H]-corticosterone-serum transcortin complex. As shown in Fig. 4, the elution profile of the cytosol-bound [^{3}H]-corticosterone in experiments with prolonged (2 h) perfusion of hearts by Krebs–Henseleit buffer solution also contained two main radioactive peaks. Position of the first peak was not changed whereas the second peak was considerably decreased and shifted to the region with lower R_F. Binding protein of the second peak appeared to differ from transcortin in sensitivity to *p*-chloromercuribenzoate and lower depression by excess of non-labelled corticosterone. Addition of non-labelled dexamethasone into incubation mixture did not influence the

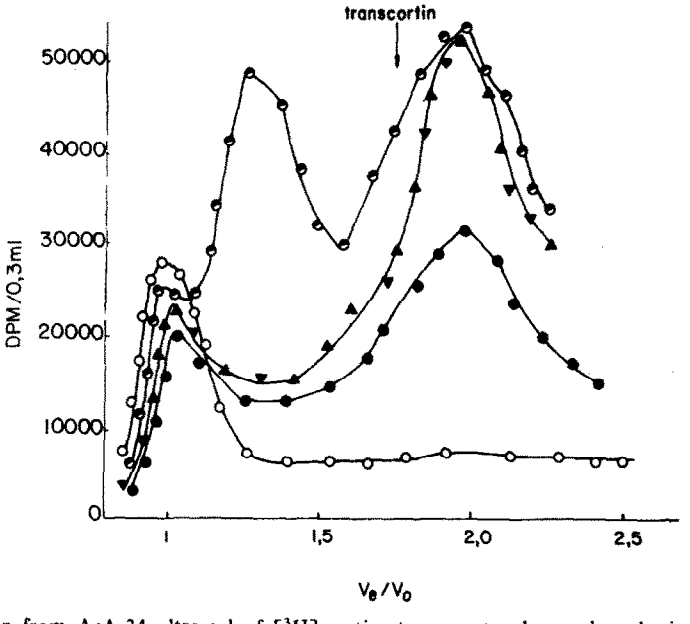

Fig. 4. Elution from AcA-34 ultra-gel of [^{3}H]-corticosterone-cytosol complex obtained from hearts after a 2 h perfusion with Krebs–Henseleit buffer solution. ◐——◐ [^{3}H]-corticosterone. ●——● [^{3}H]-corticosterone + corticosterone. ▲——▲ [^{3}H]-corticosterone + dexamethasone. ○——○ [^{3}H]-corticosterone + *p*-chloromercuribenzoate.

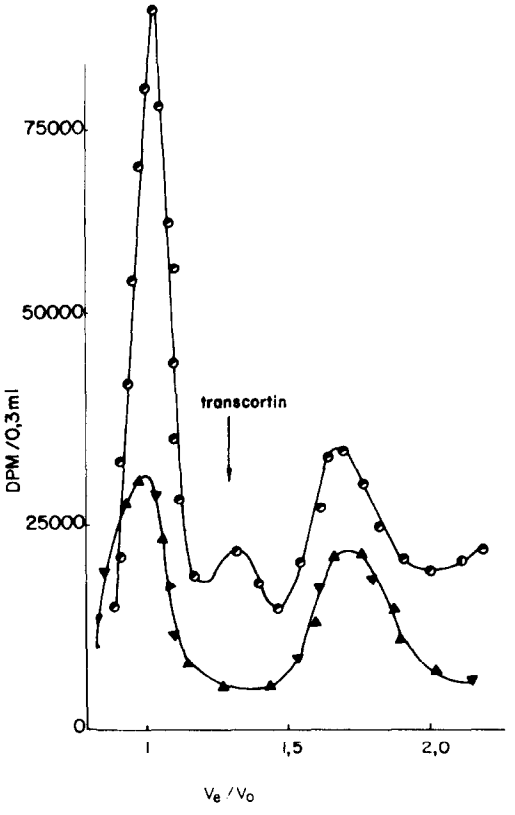

Fig. 5. Elution from AcA-44 ultra-gel of [^{3}H]-corticosterone–cytosol complex. Cytosol was obtained after a 15 min perfusion of hearts by Krebs–Henseleit solution. ○——○ [^{3}H]-corticosterone. ▲——▲ [^{3}H]-corticosterone + corticosterone.

parameters of the second peak but did sharply depress the first peak.

These results suggest that after short-term perfusion heart tissue contains remarkable amounts of transcortin, which can be removed by prolonged perfusion of the heart with Krebs–Henseleit buffer solution. After prolonged perfusion another corticosterone-binding protein (masked by a high level of transcortin) can be detected after AcA-34 ultra-gel chromatography. By using a column of AcA-44 ultra-gel and a time of heart perfusion sufficient to remove some but not all the amounts of transcortin from heart tissue, all the three types of glucocorticoid-binding proteins can be separated simultaneously. Figure 5 presents a separation of [^{3}H]-corticosterone binding fractions (on AcA-44 ultra-gel) of cytosol obtained from hearts after 15 min perfusion by Krebs–Henseleit solution. The first fraction eluted on this gel in void volume (R_F = 1) corresponds to a dexamethasone-binding protein. It also includes some amounts of non-specific aggregates. The second fraction represents transcortin remaining in tissue after 15 min perfusion. The third fraction (R_F = 1.8) is the [^{3}H]-corticosterone-binding protein which cannot be washed out of tissue after prolonged perfusion of the hearts.

The results of chromatography of [^{3}H]-corticosterone-liver cytosol complexes on AcA-44 ultra-gel (Fig. 6) show the presence of similar glucocorticoid-binding fractions in this organ. In order to find out whether other classes of steroid hormones can have higher or similar affinity to corticosterone-binding sites, as the 3d binding fraction, competitive effects of several steroids namely corticosterone, testoster-

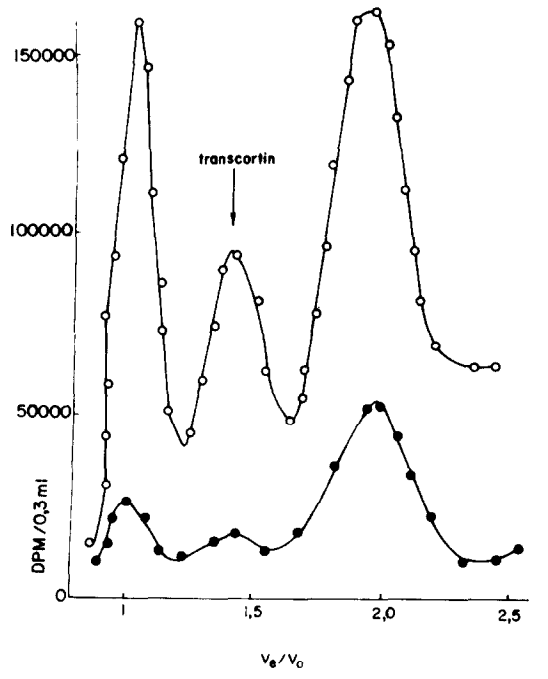

Fig. 6. Elution of [^{3}H]-corticosterone–liver cytosol complex from AcA-44 ultra-gel. ○——○ [^{3}H]-corticosterone. ●——● [^{3}H]-corticosterone + corticosterone.

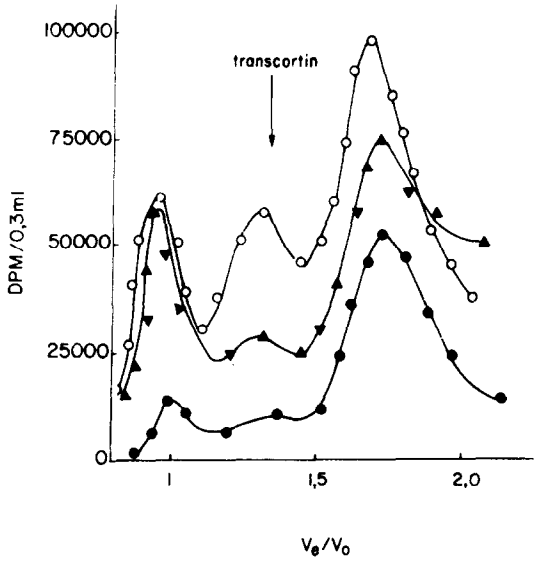

Fig. 7. The competitive effect of testosterone and corticosterone on [^{3}H]-corticosterone binding sites. Elution of [^{3}H]-corticosterone–liver–cytosol complex from a AcA-44 ultra-gel column. ○——○ [^{3}H]-corticosterone. ●——● [^{3}H]-corticosterone + corticosterone. ▲——▲ [^{3}H]-corticosterone + testosterone.

Table 1. Physical parameters of cytosol and blood serum glucocorticoid-binding fractions (as calculated from gel-chromatographic data)

	Stokes radius	Sedimentation coefficients, S	mol. wt. × 10^3 (Daltons)
Heart cytosol			
Fraction I (dexamethasone binder)			
a, 0.05 M KCl	53	7.1	191
b, 0.4 M KCl	39.5	5.25	95.5
Fraction II (transcortin)	36.5	4.55	76
Fraction III (transcortin-like)	31.5	3.55	50
Liver cytosol			
Fraction I	53	7.1	191
Fraction II	36.5	4.5	76
Fraction III	31.5	3.55	50
Serum transcortin	36.5	4.55	76

one, 5α- dehydrotestosterone, oestradiol, and progesterone on [^{3}H]-corticosterone binding by this fraction (liver cytosol) were studied. Of all hormones investigated only corticosterone was able to decrease significantly [^{3}H]-corticosterone binding. Competitive effect of 1000-fold excess of corticosterone and testosterone is shown in Fig. 7.

On the basis of calculated R_F values some physical parameters for the glucocorticoid-binding fractions from heart and liver cytosol were roughly estimated, i.e. molecular weights (according to [4]), coefficients of sedimentation (according to [5]), and Stokes radii (according to [6], Table 1). The following markers have been used for calibrating graphs: myoglobin, chymotrypsinogen, ovalbumin, bovine serum albumin, alcohol dehydrogenase, aldolase, ferritin (Serva, U.S.A.). Points on calibrating graphs corresponding to all the markers used, with the exception of ferritin, were well fitted to a straight line. Our values of molecular weights and coefficients of sedimentation for glucocorticoid complexes with liver cytosol binding fractions and serum transcortin do not completely coincide with those estimated by Koblinsky *et al.*[7], probably due to the differences in the technique used.

DISCUSSION

As it was recently shown on unfractionated heart cytosol [1, 2] binding factor I possesses high affinity for corticosterone (K_{ass} is about 2×10^8 M^{-1} at 4°C), cannot form specific complex with dexamethasone and resists heating at 37°C. Binding factor II readily binds dexamethasone (K_{ass} is about 2×10^8 M^{-1} at 4°C). Nearly the same binding affinity towards dexamethasone have been reported for glucocorticoid cytoreceptors in target organs [7, 8]. Binding factor II is highly thermolabile and inactivated after 15–20 min heating of cytosol at 37°C. Thus, using [^{3}H]-dexamethasone, only binding sites of binding factor II can be detected in intact cytosol. In cytosol preheated at 37°C [^{3}H]-corticosterone binds to the binding sites of binding factor I, whereas binding factor II in these conditions is inactivated. Several properties of binding factors I and II have been studied in unfractionated cytosol. It was demonstrated [2] that binding factor I is insensitive to SH-blocking agents, precipitated in the range 40–60% of saturation by ammonium sulphate. Binding factor II is sensitive to SH-blocking agents, precipitated in the range 0–33% of saturation by sulphate. Presence of binding factor II in cytosol is apparently required for temperature-dependent translocation of [^{3}H]-dexamethasone from cytoplasm into cell nuclei in cell-free systems [1, 2]. Inhibitory effects of various non-labelled steroids on [^{3}H]-dexamethasone-binding to unfractionated cytosol are in close relation to their glucocorticoid potencies [2].

All the data stated above shows that binding factor I is very close in properties to blood serum transcortin, whereas binding factor II in these respects is similar to the cytoreceptors of typical target organs for glucocorticoids.

Heterogeneity of the high affinity corticosterone binding sites in the unfractionated cytosol makes impossible a direct study of the naturally occurring glucocorticoids binding to the binding factor II, i.e. to the "candidate" for the glucocorticoid receptor of the heart cytoplasm. In the present work we have demonstrated the separation of the dexamethasone-binding fraction from glucocorticoid-binding proteins of other nature. The ability of this fraction to form specific complexes with [^{3}H]-corticosterone is directly shown. The dexamethasone-binding fraction separated by gel-chromatography corresponds in properties to the binding factor II of the unfractionated cytosol. Its binding activity is suppressed by excess dexamethasone or corticosterone, it is sensitive to SH-blocking agents and to heating at 37°C. Scatchard analysis of [^{3}H]-dexamethasone-binding to this separated fraction (our preliminary data) evaluates the same value of K_{ass} as for the unfractionated cytosol (2×10^8 M^{-1}). Gel-chromatography reveals that binding

factor II can exist in the two forms depending on ionic strength. At a low ionic strength it is present in cytosol in the 7.1 S form, which transforms into 5.2 S form at a high ionic strength.

Although after administration of [^{3}H]-glucocorticoids *in vivo* only 4.5–5 S forms of cytoreceptors were found by several investigators in the cytoplasm of target tissues, formation of 7–8 S specific aggregates by interaction of [^{3}H]-glucocorticoids with cytosol at low ionic strength *in vitro* has been regarded as typical behaviour of glucocorticoid cytoreceptors [9, 10]. Thus, our new data confirm the resemblance of the heart cytosol binding factor II to cytoreceptors of target tissues for glucocorticoids.

Gel-chromatography evaluates two other glucocorticoid-binding fractions sensitive to the addition of non-labelled corticosterone, both cannot bind dexamethasone and differ in size from binding factor II. The chromatographic behaviour of one of the fractions is similar to that of blood serum transcortin. Preliminary prolonged washing of the hearts by Krebs–Henseleit buffer solution leads to a significant reduction of its amount in cytosol preparations. It is therefore concluded that this fraction is the extracellular transcortin contaminating cytosol during tissue homogenization. Another fraction, "transcortin-like" (binding factor III), differs from transcortin in some respects. It has a lower R_F value, it is sensitive to SH-blocking agents and apparently has lower binding affinity and higher binding capacity towards corticosterone. Amounts of this fraction in cytosol preparations remain constant and independent of the time of washing of the heart tissue. This fact testifies to its intracellular localization. At the same time it is not clear whether this transcortin-like protein is the transcortin derivative which can enter the cells or if it is an intrinsic cellular protein. Although investigation of the structural requirements for binding was not the scope of the present work, we found in the inhibition experiments that corticosterone-binding sites of this protein possess very low affinity for non-corticoid steroids such as androgens, estrogens, progesterone.

It is interesting that specific dexamethasone-binding and transcortin-like activity have been found in cytosol preparations from a number of organs and tissues—brain [11, 12], skeletal muscle [13, 14], liver [7, 9, 10], lung [15], kidney [16]. Koblinsky *et al.*[7] were the first to separate the three specific glucocorticoid binding fractions from liver cytosol by Sephadex gel-chromatography. Later, Litwak *et al.*[10] isolated from liver cytosol five glucocorticoid binders by DEAE-chromatography, three of which were analogous to those described by Koblinsky *et al.*[7]. Our data show that three similar glucocorticoid-binding fractions can be detected in heart cytosol preparations and one of these fractions evidently represents heart cytoplasmic glucocorticoid receptor. Physiological significance of the transcortin-like protein is not clear at present.

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